Differences in the Lipoprotein Binding Profile of Halofantrine in Fed and Fasted Human or Beagle Plasma Are Dictated by the Respective Masses of Core Apolar Lipoprotein Lipid

MICHELLE P. MCINTOSH,[†] CHRISTOPHER J. H. PORTER,[†] KISHOR M. WASAN,[‡] MANISHA RAMASWAMY,[‡] AND WILLIAM N. CHARMAN*,[†]

Contribution from *Department of Pharmaceutics, Victorian College of Pharmacy, Monash University (Parkville Campus), 381 Royal Parade, Parkville, Victoria 3052, Australia, and Division of Pharmaceutics and Biopharmaceutics, Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.*

Received April 3, 1998. Final revised manuscript received November 16, 1998. Accepted for publication November 20, 1998.

Abstract □ Halofantrine hydrochloride (Hf) is an orally active, highly lipophilic antimalarial indicated for the treatment of multi-drug resistant *Plasmodium falciparum.* In this study, we have examined the binding profile of Hf to the various classes of human and beagle plasma lipoproteins as such interactions have been implicated in a post-prandial plasma lipoprotein-induced decrease in the total clearance and volume of distribution of Hf. The distribution of Hf within plasma was dominated by interaction with the various classes of plasma lipoproteins, and the characteristics and extent of binding were markedly different between species and between pre- and post-prandial plasma. In an attempt to understand the basis for the differential binding of Hf to the various lipoprotein fractions, the relationship between the proportion of Hf associated with each lipoprotein fraction (as a function of the respective mass of protein, triglyceride, cholesterol, and phospholipid) was investigated. The data indicated that the distribution of Hf between plasma lipoproteins was highly correlated with the apolar lipid load of individual plasma lipoprotein fractions suggesting that the mechanism of association was primarily via solubilization in the lipoprotein apolar lipid core. These data suggest that acute changes in plasma lipoprotein profiles, such as encountered post-prandially or in disease states such as malaria, will likely have an impact on the plasma lipoprotein binding of Hf.

Introduction

Halofantrine hydrochloride (Hf) is an orally administered, highly lipophilic phenanthrenemethanol antimalarial indicated for the treatment of acute infections caused by multi-drug resistant *Plasmodium falciparum* and *Plasmodium vivax*.[1] Absorption of Hf after fasted oral administration is poor and highly variable, although the extent of absorption is markedly enhanced when administered post-prandially.[2,3] The primary biopharmaceutical consequence of post-prandial Hf administration is improved solubilization within the bile salt rich post-prandial intestinal environment leading to enhanced absorption.[2] An interesting secondary consequence of post-prandial administration is an apparent plasma lipoprotein-induced decrease in the clearance and volume of distribution of Hf.[4]

The distribution of Hf within beagle plasma is dominated by interaction with plasma lipoproteins, with the characteristics and extent of the binding reflecting the relative pre- and post-prandial lipoprotein profiles.[4,5] In beagles, a significant temporal relationship was noted between the association of Hf with lipoproteins and the post-prandial reduction in total clearance (15%) and volume of distribution (21%).[4] This effect was most likely mediated via a reduction in the free fraction of Hf in plasma which occurred secondarily to increased binding to plasma lipoproteins.[4]

The profile of plasma lipoproteins varies widely between and within individuals as a function of age, gender, co-administered drugs, diet, and disease states including alcoholism, diabetes, and acute malaria,[6,7] thereby raising the possible specter of altered therapeutic profiles of highly lipoprotein-bound drugs (such as Hf) under these circumstances.[8] For example, it was recently demonstrated that the IC_{50} of Hf determined in continuous in vitro culture of *P. falciparum* was significantly increased when incubated in the presence of 10% post-prandial serum.[9] From a drug targeting standpoint, as high-density lipoproteins (HDL) are a major phospholipid source which can support the intra-erythrocytic stage of parasite reproduction,[10] the possibility of HDL-associated uptake of Hf into infected erythrocytes has been suggested.[11] From a toxicological standpoint, there appears to be a linkage between excessively high plasma Hf concentrations and cardiac side effects (typically observed as a lengthening of the QTc interval) in patients with a preexisting cardiopathy.[12] As excessively high plasma concentrations of Hf are typically observed after post-prandial administration, elucidation of the distribution of Hf between post-prandial lipoproteins may contribute to a better understanding of the possible factors contributing to the changed QTc profiles.

In this study, we have extended our previous preliminary examination[5] of the in vivo lipoprotein binding of Hf in beagle plasma to pre- and post-prandial human plasma. In an attempt to understand the basis for the differential binding of Hf across lipoprotein fractions, the relationship between the proportion of Hf associated with each lipoprotein fraction (as a function of the respective mass of protein, triglyceride, cholesterol and phospholipid) was investigated between species in pre- and post-prandial states.

Materials and Methods

Chemicals—Halofantrine hydrochloride and Hf base were obtained from SmithKline Beecham Pharmaceuticals (Brentford, Middlesex, U.K.). Acetonitrile (Mallinckrodt, Paris, KY) and *tert*-butyl methyl ether (Fluka, Buchs, Switzerland) were HPLC grade, and sodium dodecyl sulfate (SDS, Eastman Kodak, Rochester, NY) was electrophoresis grade. All other chemicals were at least AR grade, and water was obtained from a Milli-Q (Millipore, Bedford, MA) water purification system.

* To whom correspondence should be addressed. Phone: +61 3 9903 9519. Fax: +61 3 9903 960. E-mail: bill.charman@vcp.monash.edu.au.
[†] Monash University.
[‡] The University of British Columbia.

10.1021/js980152g CCC: $18.00 *Published on Web 01/23/1999*
© 1999, American Chemical Society and American Pharmaceutical Association

Plasma Incubation Studies—Blank plasma was taken from healthy male beagle dogs after either an overnight fast or 4 h after ingestion of 400 g of standard canned dog food (average composition: 2.5% fat, 7.5% protein) supplemented with 50 mL of soybean oil (a representative long-chain triglyceride). Human plasma was obtained from a healthy male volunteer after either an overnight fast or 4 h after ingestion of a lipid rich meal (fat content greater than 50 g: MacDonalds Big Mac and french fries). Blood samples were collected into sterile tubes, anti-coagulated with 1.5 mg mL^{-1} Na_2EDTA, and centrifuged at 2000 rpm for 15 min, and the plasma stored at 4−8 °C. For incubation studies, fresh blank plasma (used within 3 h of collection) was prewarmed to 37 °C and spiked with drug dissolved in ethanol (10 μL of ethanol per mL of plasma) to provide a final drug concentration of 1000 ng mL^{-1} (Hf base equivalents). The addition of up to 50 μL of ethanol per mL of plasma does not have a discernible affect on plasma lipoprotein concentrations or their respective composition or integrity.[13] The spiked plasma samples were incubated at 37 °C for 60 min in a temperature-controlled water bath, and the samples were gently swirled manually at 5 min intervals. At the conclusion of the incubation period, samples were placed in an ice bath for at least 5 min prior to immediate density gradient ultracentrifugation.

The Hf distribution profiles from the in vitro spiking studies were compared with the distribution profile of Hf in a plasma sample taken from a fasted male beagle (3 yr, 18 kg) 30 min after intravenous administration of a 2 mg kg^{-1} dose of Hf base prepared in Intralipid.[14]

Lipoprotein Separation—After incubation of plasma with Hf, 1 mL plasma samples were fractionated by single spin density gradient ultracentrifugation as previously described.[5,15] Briefly, stock buffer for density gradient solutions contained 0.1% NaN_3, 0.2% Na_2EDTA, and 1.2% Tris base dissolved in distilled water adjusted to pH 7.4, which was stored at room temperature after filtration. Density gradient solutions were prepared by adding appropriate quantities of KBr to a plasma background solution, and the solution densities were confirmed using refractometry. The density gradient was formed in a 4 mL centrifuge tube (Seton Scientific, Sunnyvale, CA), and the samples were centrifuged at 15 °C for 19 h at 58 000 rpm (Sorvall OTD65B Ultracentrifuge, Newtown, CT) using a SW 60 rotor (Beckman, CA). Plasma samples were separated into the following fractions: triglyceride rich lipoproteins (TRL, which includes VLDL and chylomicrons), low-density lipoproteins (LDL), high-density lipoproteins (HDL), and lipoprotein deficient plasma (LPDP). In a minor modification to our previous technique,[4,5] the majority of TRL was first recovered from the top of the centrifuge tube by needle aspiration of 2 × 200 μL aliquots. This modification improved the separation of post-prandial TRL and minimized potential contamination of the more dense fractions with TRL. The LDL, HDL, and the LPDP fractions were then obtained using a fraction collection system (Beckman, CA) where a high-density solution (Fluorinert, Sigma Chemicals, St Louis, MO) was pumped into the bottom of each centrifuge tube at 24 mL h^{-1}, the eluent was collected from the top of the tube and passed through a UV detector set at 280 nm (to identify peak elution via monitoring of lipoprotein-associated protein), prior to the automated collection of 18 × 200 μL aliquots.

Each aliquot was analyzed for total protein (TP), total cholesterol (CH), free cholesterol (FC), triglyceride (TG), and phospholipid (PL) using commercial enzymatic colorimetric kits (Bohringer Mannheim, Germany) running on a Cobas BIO clinical analyzer (Roche, Basle, Switzerland). The analyzer was externally calibrated for each run and quality control samples were run on a weekly basis. In aliquots from beagle plasma, the amount of cholesteryl ester (CE) was calculated as the difference between the measured CH and FC values, whereas in aliquots from human plasma the mass of CE was calculated as a fixed percentage of the measured TC value for each individual lipoprotein fraction.[16]

The individual cholesterol and triglyceride concentration profiles in each aliquot were used to define the respective TRL, LDL, HDL, and LPDP fractions. Typically, the two aspirated aliquots were combined with the initial two aliquots obtained using the fraction recovery system (i.e., aliquots 3 and 4) and designated as the TRL fraction, aliquots 5−11 were combined for the LDL fraction, aliquots 12−17 were combined for the HDL fraction, and aliquots 18−20 were combined for the LPDP fraction.

Analysis of Hf in Lipoprotein Fractions—The concentration of Hf in plasma and individual lipoprotein fractions was determined using a validated assay.[17] Due to the logistical difficulty in obtaining sufficient quantities of blank lipoproteins to routinely prepare standard curves for each individual lipoprotein fraction, spiked blank plasma was used to prepare standard curves on a daily basis. The validity of this approach was confirmed by the similarity in the ratio of the peak area/concentration values (<5%) from standard curves prepared using either plasma or specifically isolated lipoprotein.

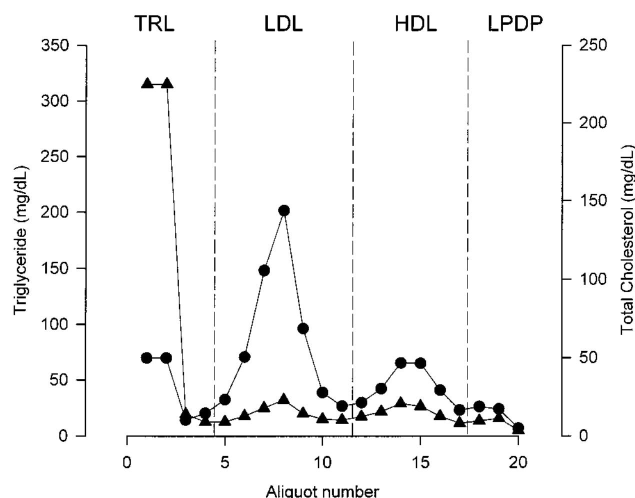

Figure 1—Representative profile of total cholesterol (●) and triglyceride (▲) concentrations as a function of fractionation aliquot obtained after density gradient ultracentrifugation of 1 mL of post-prandial human plasma. Aliquots 1–4 were combined for the TRL fraction, aliquots 5–11 for the LDL fraction, aliquots 12–17 for the HDL fraction, and aliquots 18–20 were designated as the LPDP fraction.

Compositional Analysis of LP Fractions—To explore the basis for the distribution of Hf between lipoprotein fractions, the compositional profile of individual lipoprotein fractions based on measured PL, CH, FC, CE, TG, and TP values were assessed in the following manner. The mass of apolar lipid, which effectively constitutes the hydrophobic core of lipoproteins, was calculated for each respective fraction as the sum of the CE and TG content. The volume of the hydrophobic core was calculated assuming individual lipoprotein particles to be spherical, with each lipoprotein fraction having a similar gross structure including a 20.5 Å thick surface monolayer as previously described.[18] The following average values[16] for the density (ρ) and average radius (r) of human lipoproteins were used to calculate the volume of the hydrophobic core and the surface area of individual human lipoprotein fractions: VLDL, r = 275 Å, ρ = 989 mg mL^{-1}; LDL, r = 100 Å, ρ = 1042 mg mL^{-1}; HDL, r = 37.7 Å, ρ = 1136.5 mg mL^{-1}. The volume and surface area values of lipoprotein fractions from beagle plasma could not be estimated as published density and radius data were not available.

Results

The lipoprotein fraction isolation procedure employed in the current study was modified to better separate the increased quantities of TRL present in post-prandial plasma compared with our previous technique designed for pre-prandial plasma.[4,5] Figure 1 depicts a typical separation profile of post-prandial human plasma when monitored using the TG and TC concentrations present in individual fractionation aliquots. Importantly, there was distinct separation of TG associated with the sequential TRL and LDL fractions indicating an excellent separation profile.

The distribution of Hf between lipoprotein fractions obtained after in vitro spiking of pre-prandial beagle plasma with an ethanolic Hf·HCl solution, or from a blood sample taken from a pre-prandial beagle after intravenous administration of Hf base (sample taken at 30 min post-dosing as this afforded a plasma Hf concentration of approximately 1000 ng mL^{-1}), are presented in Table 1.

Table 1—Percentage Distribution of Hf between Plasma Lipoprotein Fractions[a] (Mean ± SD, $n = 3$) Obtained after in Vitro Incubation of Fasted Beagle Plasma with Hf•HCl Dissolved in Ethanol (10 μL/mL Plasma) and from an in Vivo Plasma Sample Taken from a Fasted Beagle 30 min after Intravenous Administration of 2 mg kg^{-1} Hf Base

treatment	TRL	LDL	HDL	LPDP	recovery (%)[b]
in vitro incubation of fasted plasma with Hf•HCl	3.3 ± 0.9	10.1 ± 0.8	51.2 ± 4.4	35.4 ± 2.7	93.8 ± 3.7
in vivo plasma sample after IV administration of Hf base	3.2 ± 0.5	8.9 ± 0.4	52.3 ± 1.2	35.2 ± 1.4	101.3 ± 1.4

[a] TRL, triglyceride rich lipoproteins which include VLDL and chylomicrons; LDL, low-density lipoproteins; HDL, high-density lipoproteins; LPDP, lipoprotein deficient plasma. [b] Recovery is defined as the % mass of drug recovered after plasma fractionation divided by the mass of drug present in the original sample (as either the in vitro spike or the plasma Hf concentration in the in vivo sample).

Table 2—Percentage Distribution of Halofantrine (Hf) between Plasma Lipoprotein Fractions[a] (Mean ± SD, $n = 3$) Obtained after in Vitro Incubation of 1000 ng mL^{-1} Hf•HCl with Plasma Obtained from Pre- and Post-prandial Human Subjects, and Pre- and Post-prandial Beagles

lipoprotein fraction	percentage distribution of Hf			
	in human plasma		in beagle plasma	
	pre-prandial	post-prandial	pre-prandial	post-prandial
TRL	17.3 ± 1.2	39.7 ± 1.2[b]	3.3 ± 0.9	26.6 ± 2.0[b]
LDL	31.5 ± 0.2	18.6 ± 1.0[b]	10.1 ± 0.8	7.5 ± 1.1[b]
HDL	6.1 ± 0.6	6.5 ± 2.4	51.2 ± 4.4	37.5 ± 0.9[b]
total LP binding	54.8 ± 0.9	64.8 ± 3.2[b]	64.6 ± 2.7	71.5 ± 1.4[b]
LPDP	45.2 ± 0.9	35.2 ± 3.2[b]	35.4 ± 2.7	28.5 ± 1.4[b]
% recovery[c]	99.1 ± 4.4	109.1 ± 1.6	93.8 ± 3.7	90.2 ± 4.0

[a] TRL, triglyceride rich lipoproteins which include VLDL and chylomicrons; LDL, low-density lipoproteins; HDL, high-density lipoproteins; LPDP, lipoprotein deficient plasma. [b] Significantly different to the corresponding pre-prandial value ($p < 0.05$). [c] Recovery is defined as the % mass of drug recovered after plasma fractionation divided by the mass of drug present in the original sample.

Greater than 90% of Hf present in either plasma sample was accounted for in the TRL, LDL, HDL, and LPDP fractions. The utility of the in vitro spiking procedure was confirmed by the similar distribution profiles of Hf between the various lipoprotein fractions isolated from either the in vitro or in vivo samples.

Table 2 describes the lipoprotein distribution of Hf in spiked pre- and post-prandial human and beagle plasma when incubated with Hf using the validated in vitro procedure. The distribution of Hf in beagle and human plasma was dominated by significant binding to lipoproteins as 54.8% of Hf was lipoprotein bound in fasted human plasma, and 64.6% was lipoprotein bound in fasted beagle plasma. Additionally, there were increases in the extent of Hf binding by lipoproteins in pre-prandial compared with the post-prandial state (an 18.2% increase in human plasma, and a 10.7% increase in beagle plasma).

Although the extent of lipoprotein binding of Hf was similar between man and dog, analysis of the distribution of Hf between individual lipoprotein fractions indicated substantial species differences. For example, a much larger proportion of the plasma concentration of Hf was carried in TRL and LDL fractions in human plasma compared with beagle plasma, whereas the proportion of Hf carried in HDL was 6–8-fold higher in beagle plasma compared with human plasma (Table 2).

Assessment of the distribution of Hf between pre- and post-prandial lipoprotein classes demonstrated increased amounts of drug associated with the lipid rich TRL fraction of post-prandial plasma with an average 2-fold increase observed in human plasma, and a 8-fold increase in beagle plasma. In contrast, there were decreases in the proportion of Hf associated with post-prandial LDL and LPDP fractions in human and beagle plasma, and HDL in beagle plasma. The proportion of Hf in human HDL was low (6%) and apparently unchanged in pre- and post-prandial human, whereas a much larger proportion of Hf was associated with HDL in pre-prandial beagle plasma (51%) which decreased in the post-prandial state.

The mass of TG, CH, FC, CE, PL, and TP determined in each lipoprotein fraction isolated from the Hf spiked pre- and post-prandial human and beagle plasma are presented in Table 3, with the values for human lipoproteins being similar to literature reports.[19] Data are also presented describing the individual quantities of apolar lipid (i.e. TG + CE) and total lipid (TC + TG + PL) present in the various isolated lipoprotein fractions.

Discussion

In a preliminary communication, we demonstrated that Hf was highly associated with lipoproteins in beagle plasma, and that the characteristics of association were altered when the concentration profile of plasma lipids increased post-prandially.[5] The primary change was an increased proportion of Hf associated with TRL in post-prandial plasma, and from a pharmacokinetic standpoint, this was accompanied by a reduction in drug clearance and volume of distribution which most likely arose through reduction in the plasma free fraction of drug.[4] In this study, we have examined the association of Hf with human plasma lipoproteins (as a possible indicator of similar effects in humans) and explored correlations between the association of Hf with lipoproteins and the mass of protein, triglyceride (TG), cholesterol (CH), and phospholipid (PL) within specific lipoprotein classes as a possible predictor of the basis for the interaction.

It was only possible to study the association of Hf with human plasma lipoproteins in an in vitro setting as an acceptable intravenous formulation for human administration was not available,[20] and potential toxicity concerns precluded post-prandial oral administration to healthy volunteers.[12] Consequently, it was necessary to demonstrate the equivalence of a plasma spiking methodology which afforded a Hf lipoprotein binding profile representative of the in vivo situation.

Employing beagles as a test species, the data in Table 1 indicates that the binding profile of Hf across lipoprotein fractions in plasma sampled after intravenous drug administration was essentially identical to that obtained after fractionation of plasma spiked with Hf, thereby indicating the equivalence of the spiking methodology. Similar in vivo and in vitro lipoprotein distribution profiles have been reported for cyclosporin when small volumes of ethanol were used as the plasma spiking solvent.[21]

Application of this spiking methodology to pre- and post-prandial human plasma, and subsequent fractionation of plasma into TRL, LDL, HDL, and LPDP subgroups, indicated that Hf was also significantly bound to pre- and post-prandial human plasma lipoproteins although the distribution profile was markedly different to that observed in beagles (Table 2). Broadly, the proportion of Hf associated with the TRL and LDL fractions was higher in humans compared with beagles, whereas the proportion of Hf associated with HDL was much higher in beagles. These findings reflect the broad species differences in lipoprotein profiles (Table 3) where a larger proportion of human plasma lipid is carried by TRL and LDL fractions,

Table 3—Quantities of Plasma Lipids and Protein from Isolated Lipoprotein Fractions (Mean ± SD, $n = 3$) in Pre- and Post-prandial Human and Beagle Plasma (Lipoprotein Fractions Are the Same as Those Used To Determine the Distribution of Hf Described in Table 2)

	human plasma		beagle plasma	
lipoprotein composition (mg mL^{-1})	pre-prandial	post-prandial	pre-prandial	post-prandial
TRL fraction				
total cholesterol	0.20 ± 0.01	0.27 ± 0.02[a]	0.05 ± 0.00	0.07 ± 0.00[a]
cholesterol ester	0.13 ± 0.01	0.17 ± 0.01[a]	0.05 ± 0.00	<0.05
free cholesterol	0.07 ± 0.00	0.09 ± 0.01[a]	<0.05	0.11 ± 0.00[a]
triglyceride	0.38 ± 0.05	1.38 ± 0.05[a]	0.12 ± 0.00	0.87 ± 0.02[a]
phospholipid	0.11 ± 0.07	0.20 ± 0.07	0.04 ± 0.00	0.14 ± 0.00[a]
protein	<0.15	<0.15	<0.15	<0.15
apolar lipid (TG + CE)	0.51 ± 0.06	1.55 ± 0.06[a]	0.17 ± 0.00	0.84 ± 0.01[a]
total lipid (TC + TG + PL)	0.68 ± 0.12	1.86 ±0.14[a]	0.21 ± 0.00	1.09 ± 0.02[a]
apolar lipid/total plasma lipid (%)	15.43 ± 1.64	36.38 ± 1.44[a]	3.28 ± 0.04	13.10 ± 0.19[a]
apolar lipid/plasma apolar lipid (%)	25.33 ± 1.41	52.05 ± 2.53[a]	10.70 ± 0.40	35.50 ± 1.71[a]
LDL fraction				
total cholesterol	0.89 ± 0.06	0.86 ± 0.02	0.24 ± 0.00	0.23 ± 0.00
cholesterol ester	0.72 ± 0.05	0.69 ± 0.02	0.18 ± 0.00	0.19 ± 0.00
free cholesterol	0.17 ± 0.01	0.17 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
triglyceride	0.29 ± 0.00	0.24 ± 0.03	0.14 ± 0.01	0.17 ± 0.01
phospholipid	0.41 ± 0.17	0.30 ± 0.07	0.25 ± 0.01	0.26 ± 0.02
protein	0.20 ± 0.20	0.20 ± 0.00	<0.15	<0.15
apolar lipid (TG + CE)	1.01 ± 0.05	0.93 ± 0.05	0.32 ± 0.01	0.35 ± 0.01
total lipid (TC + TG + PL)	1.59 ± 0.21	1.40 ± 0.08	0.63 ± 0.01	0.66 ± 0.03
apolar lipid/total plasma lipid (%)	30.72 ± 2.93	21.84 ± 1.94	6.09 ± 0.22	5.53 ± 0.13
apolar lipid/plasma apolar lipid (%)	50.41 ± 1.12	31.18 ± 1.12[a]	19.90 ± 0.29	15.00 ± 0.84[a]
HDL fraction				
total cholesterol	0.40 ± 0.03	0.37 ± 0.02	1.41 ± 0.06	1.32 ± 0.03
cholesterol ester	0.32 ± 0.02	0.30 ± 0.02	1.09 ± 0.06	1.00 ± 0.02
free cholesterol	0.08 ± 0.00	0.07 ± 0.00	0.31 ± 0.00	0.33 ± 0.01
triglyceride	0.16 ± 0.01	0.21 ± 0.04	0.04 ± 0.00	0.17 ± 0.13
phospholipid	0.47 ± 0.12	0.46 ± 0.11	3.03 ± 0.07	3.16 ± 0.05
protein	2.20 ± 0.92	2.07 ± 0.81	3.43 ± 0.08	3.21 ± 0.05
apolar lipid (TG + CE)	0.48 ± 0.03	0.50 ± 0.05	1.13 ± 0.07	1.17 ± 0.10
total lipid (TC + TG + PL)	1.03 ± 0.14	1.03 ± 0.10	4.47 ± 0.05	4.66 ± 0.05
apolar lipid/total plasma lipid (%)	14.78 ± 1.33	11.77 ± 1.61	21.20 ± 1.20	18.30 ± 1.61
apolar lipid/plasma apolar lipid (%)	24.26 ± 0.29	16.78 ± 1.42[a]	69.40 ± 0.65	49.50 ± 2.54[a]
whole plasma				
total lipid (TC + TG + PL)	3.30 ± 0.46	4.28 ± 0.28	5.31 ± 0.07	6.40 ± 0.02[a]
total apolar lipid (TG + CE)	2.00 ± 0.14	2.99 ± 0.07[a]	1.63 ± 0.08	2.36 ± 0.09[a]

[a] Significantly different to the corresponding pre-prandial value ($p < 0.05$).

whereas HDL are the major lipid carrier in dogs. The post-prandial state induced significant changes in the distribution of Hf between plasma lipoprotein fractions, with increased proportions of Hf in the TRL fractions in both human and beagle plasma at the expense of a species-dependent decrease in the proportion of Hf in LDL and HDL fractions (Table 2). Unfortunately, toxicity concerns and related ethical issues precluded our assessment of the post-prandial pharmacokinetics of Hf in human volunteers. However, as the in vitro studies identified increased post-prandial lipoprotein binding of Hf in human plasma, this suggests the possibility of a potential decrease in clearance and volume of distribution (as observed in the beagle studies).[4]

Historically, methods used to quantify drug–lipoprotein interactions have been modifications of classical protein binding experiments. Typically, individual association constants (K) and the number of binding sites (n) associated with drug–lipoprotein binding are generated from study of the interaction between a drug substrate and isolated fractions using Scatchard-type analyses. Subsequently, an indication of the overall lipoprotein binding profile is obtained from the product of the affinity constant, the apparent number of binding sites and the estimated concentration of each lipoprotein class in plasma.[22] The lipoprotein binding profile of drugs such as anthracycline,[23] ticlopidine and PCR2362,[24] nicardipine,[25] propranolol,[22] and etretinate and acitretin[26] have been assessed in this manner. In contrast to the majority of drug–plasma protein interactions, the nature of the lipoprotein association profiles for these compounds is nonsaturable, which only allows for calculation of the overall binding capacity from the product of the association constant and the number of binding sites. In each of the above examples, the order of the lipoprotein binding capacity (expressed per mole of lipoprotein) was VLDL > LDL > HDL, and the binding was seemingly related to the size and/or overall lipid content of the lipoprotein subclasses. In each case, it was suggested that the nonsaturable nature of the binding, and the apparent correlation of the binding capacity with the size and/or lipid content of the lipoproteins indicated that the binding interaction was solubilization-based rather than a specific binding phenomenon.

To investigate solubilization as the basis for the interaction of Hf with the various lipoprotein fractions, correlations were investigated between the amount of Hf present in individual fractions and the corresponding mass of total protein, phospholipid, triglyceride, and apolar lipid in human and beagle plasma lipoproteins (Figures 2 and 3, respectively). In beagle and human plasma, the Hf distribution profile was poorly correlated with individual lipoprotein surface constituents (protein and phospholipid), reasonably correlated with TG profiles in human compared with beagle lipoproteins, and better correlated with the mass of the apolar lipid core (TG + CE) in both human and beagle lipoprotein fractions.

As representative dimensions were available for the various lipoprotein fractions in human plasma,[16] this

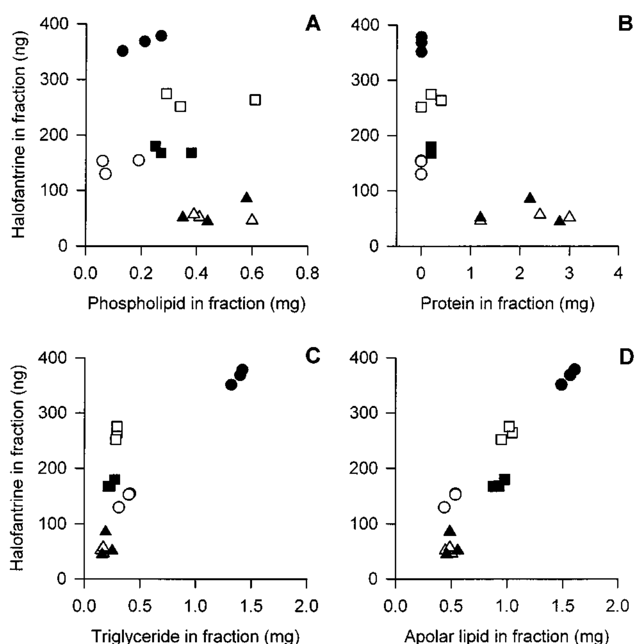

Figure 2—Correlation between the mass of Hf present in pre-prandial (open symbols) and post-prandial (closed symbols) HUMAN plasma lipoprotein fractions and the mass of specific lipoprotein components present in the individual TRL (●); LDL (■), and HDL (▲) fractions. (A) Correlation with the mass of phospholipid per fraction ($r^2 = 0.14$). (B) Correlation with the mass of protein per fraction ($r^2 = 0.50$). (C) Correlation with the mass of triglyceride per fraction ($r^2 = 0.66$). (D) Correlation with the mass of apolar lipid per fraction ($r^2 = 0.88$).

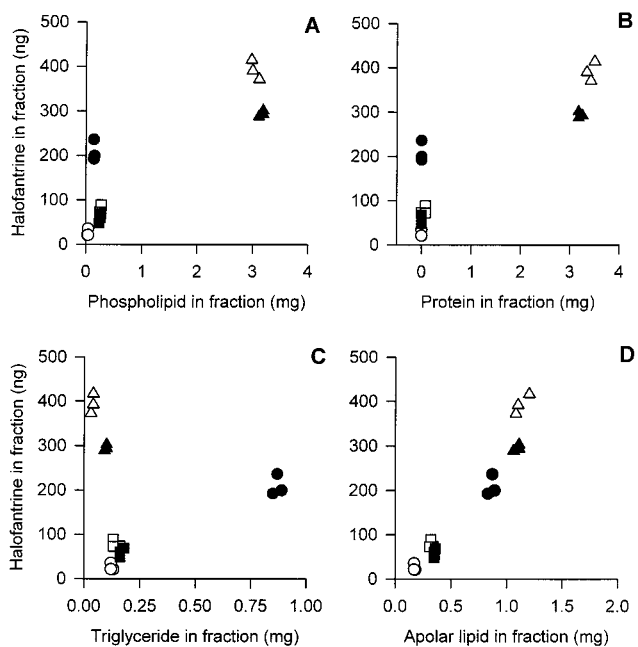

Figure 3—Correlation between the mass of Hf present in pre-prandial (open symbols) and post-prandial (closed symbols) BEAGLE plasma lipoprotein fractions and the mass of specific lipoprotein components present in the individual TRL (●); LDL (■) and HDL (▲) fractions. (A) Correlation with the mass of phospholipid per fraction ($r^2 = 0.79$). (B) Correlation with the mass of protein per fraction ($r^2 = 0.81$). (C) Correlation with the mass of triglyceride per fraction ($r^2 = 0.01$). (D) Correlation with the mass of apolar lipid per fraction ($r^2 = 0.93$).

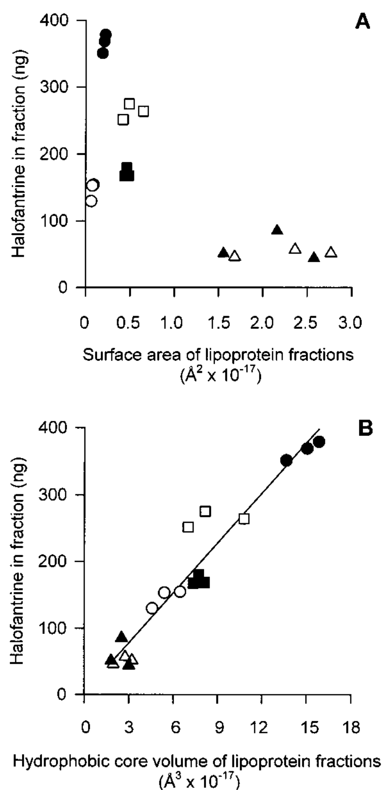

Figure 4—Correlation between the mass of Hf present in individual pre-prandial (open symbols) and post-prandial (closed symbols) HUMAN lipoprotein fractions (TRL (●), LDL (■), HDL (▲)) and the calculated surface area of the individual lipoprotein fractions ((A) $r^2 = 0.52$) and the calculated volume of the hydrophobic core of the individual lipoprotein fractions ((B) $r^2 = 0.93$).

enabled estimation of the surface area and hydrophobic core volume parameters for each lipoprotein fraction in human plasma. Figure 4 presents the relationship between the mass of Hf present in each fraction and these calculated parameters. There was a poor correlation with the respective surface area of individual fractions, but an improved correlation with the calculated volume of the hydrophobic core of the lipoprotein fractions.

The positive correlation between the mass of Hf in each lipoprotein fraction and the mass of apolar lipid as depicted in Figures 2 and 3, and the calculated volume of the hydrophobic core presented in Figure 4, was consistent with solubilization of Hf in the apolar lipid core and inconsistent with a specific interaction with surface groups or apolipoproteins. Furthermore, the correlation parameters were similar for all lipoprotein groups in human and beagle plasma (Figures 2 and 3), indicating that the binding/solubilization of Hf was independent of the lipoprotein fraction, relative lipid load, and species. These data indicated that the quantity of Hf solubilized per mg of lipoprotein apolar lipid was effectively constant regardless of the overall lipoprotein composition. On first inspection, these data were surprising as significant compositional differences exist between TRL, LDL, and HDL and across species. However, although the percentage composition of lipoproteins varies with the changing particle sizes (e.g. smaller lipoproteins such as HDL have proportionally more surface material such as PL and protein), the nature of the hydrophobic apolar core lipids is largely invariant such that their capacity to solubilize Hf was proportional to the respective mass of apolar lipid.

These data suggest that the trend governing the association of Hf with lipoprotein fractions was the quantity of apolar lipid in each of the respective fractions. Although the decreased proportion of Hf present in post-prandial LDL compared with pre-prandial LDL (despite the mass of LDL apolar lipid remaining relatively constant) (Tables 2 and 3) appears to contradict this scenario (Table 2), the actual driving force behind the lipoprotein distribution of Hf was the *proportional* distribution of lipids across the

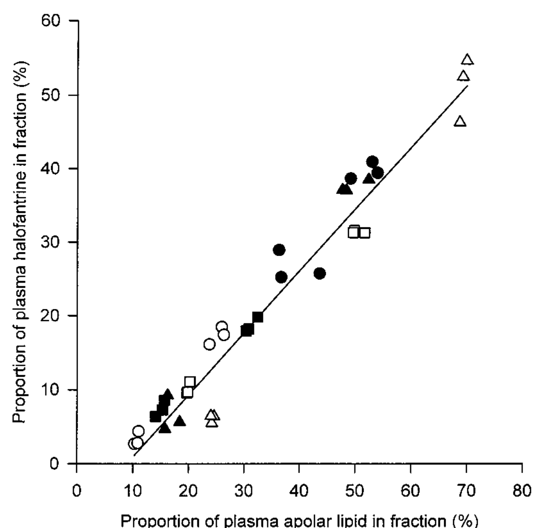

Figure 5—Combined correlation between the proportion of Hf distributed between lipoprotein fractions (TRL (●), LDL (■), HDL (▲)) in human and beagle plasma and the proportional distribution of apolar lipid (TG + CE) within the individual lipoprotein fractions from pre-prandial (open symbols) and post-prandial plasma (closed symbols). The correlation coefficient was 0.95.

lipoprotein fractions and not their absolute masses. Therefore, although the mass of apolar lipid in LDL was similar between pre- and post-prandial states, the proportion of apolar plasma lipid carried by LDL decreased due to the increased quantity of apolar lipid present in the TRL fraction. From consideration of the proportional distribution of apolar lipid, Figure 5 presents the combined correlation between the proportion of Hf between lipoprotein fractions from pre- and post-prandial beagle and human plasma and the proportional distribution of apolar lipid across the individual fractions. The significant correlation between these parameters across species, lipoprotein fractions, and pre- and post-prandial states supports the contention that the apolar lipid content of the individual lipoprotein fractions is the basis for the lipoprotein distribution of Hf.

For drugs with significant lipoprotein binding, the overall binding profile can be estimated from the product of the binding capacity per mole (nK) and the concentration of each individual lipoprotein fraction in plasma. Therefore, as the relative concentrations of plasma lipoprotein fractions are VLDL ($\approx$0.1 μM) < LDL ($\approx$1 μM) < HDL ($\approx$11 μM),[23,24,26] the distribution profile of lipophilic drugs often reflects the relative lipoprotein concentrations such that a larger proportion of the drug is carried by HDL or LDL as observed for ticlopidine,[24] nicardipine,[25] diclofenac,[27] and propranolol.[22] Employing this approach, Cenni and co-workers[28] recently determined the in vitro binding constants (nK) of Hf to human serum constituents (LDL and HDL, human serum albumin α_1-acid glycoprotein) and predicted that a significant proportion of Hf in plasma would be associated with lipoproteins. On the basis of the calculated binding constants, Cenni and co-workers predicted that approximately 67% of Hf in serum would be associated with LDL, about 25% with HDL and that the unbound fraction of Hf in blood would be as low as 0.4%. The marked differences between the data reported in this study and the predicted Hf distributions described by Cenni and co-workers[28] are likely to be (at least in part) a result of an erroneous assumption of minimal Hf binding to TRL. This assumption was based on results of preliminary gel filtration chromatography studies for Hf binding to serum constituents where the recovery of Hf was only 40%. Therefore, the low recovery of Hf from the preliminary gel filtration studies may have clouded the accuracy of the binding profile such that significant binding of Hf with constituents such as TRL was not observed. We believe that the direct method of estimating the plasma lipoprotein binding profile of Hf in plasma employed in the current study yields a more accurate estimate of the overall lipoprotein binding and plasma distribution of Hf.

In summary, the results from this study extends to human plasma the previously identified changes in the post-prandial distribution of Hf in beagle plasma, and raises the possibility of a decrease in Hf clearance and volume of distribution in post-prandial compared with pre-prandial human subjects. The data from this study indicate that the distribution of Hf among plasma lipoproteins was highly correlated with the apolar lipid load of individual plasma lipoprotein fractions thereby suggesting that the mechanism of Hf association with plasma lipoprotein was primarily via solubilization in the apolar lipid core. Furthermore, these findings suggest that acute changes in plasma lipoprotein distributions, such as encountered post-prandially or in disease states such as malaria, may markedly affect the plasma lipoprotein binding of Hf (and other highly lipophilic drugs).

References and Notes

1. Horton, J.; Parr, S. N.; Bokor, L. C. Clinical experience with halofantrine in the treatment of malaria. *Drugs Exp. Clin. Res.* **1990**, *16*, 497–503.
2. Humberstone, A. J.; Porter, C. J. H.; Charman, W. N. A physicochemical basis for the effect of food on the absolute oral bioavailability of halofantrine. *J. Pharm. Sci.* **1996**, *85*, 525–529.
3. Milton, K. A.; Edwards, G.; Ward, S. A.; Orme, M. L.; Breckenridge, A. M. Pharmacokinetics of halofantrine in man: effects of food and dose size. *Br. J. Clin. Pharmacol.* **1989**, *28*, 71–77.
4. Humberstone, A. J.; Porter, C. J. H.; Edwards, G. A.; Charman, W. N. Association of halofantrine with post-prandially derived plasma lipoproteins decreases its clearance relative to administration in the fasted state. *J. Pharm. Sci.* **1998**, *87*, 936–942.
5. Humberstone, A. J.; Porter, C. J. H.; Edwards, G.; Charman, W. N. Distribution of halofantrine between plasma lipoprotein fractions after IV administration to pre- and post-prandial beagle dogs. *Pharm. Res.* **1995**, *12*, S-356 (abstract).
6. Schaefer, E. J.; Levy, R. I. Pathogenesis and management of lipoprotein disorders. *N. Engl. J. Med.* **1985**, *312*, 1300–1310.
7. Mohanty, S.; Mishra, S. K.; Das, B. S.; Satpathy, S. K.; Mohanty, D.; Patnaik, J. K.; Bose, T. K. Altered plasma lipid pattern in *falciparum* malaria. *Ann. Trop. Med. Parasitol.* **1992**, *86*, 601–606.
8. Wasan, K. M.; Cassidy, S. M. Role of plasma lipoproteins in modifying the biological activity of hydrophobic drugs. *J. Pharm. Sci.* **1998**, *87*, 411–424.
9. Humberstone, A. J.; Cowman, A. F.; Horton, J.; Charman, W. N. Effect of altered serum lipid concentrations on the IC_{50} of halofantrine against *Plasmodium falciparum*. *J. Pharm. Sci.* **1998**, *87*, 256–258.
10. Grellier, P.; Rigomier, D.; Clavey, V.; Fruchart, J. C.; Schrevel, J. Lipid traffic between high-density lipoproteins and *Plasmodium falciparum*-infected red blood cells. *J. Cell Biol.* **1991**, *112*, 267–277.
11. Cenni, B. *Plasmodium falciparum*: Characteristics of halofantrine accumulation in vitro in parasitised red blood cells. Ph.D. Thesis, Swiss Tropical Institute, Basle, Switzerland, 1994,
12. Monlun, E.; Le Metayer, P.; Szwandt, S.; Neau, D.; Longy Boursier, M.; Horton, J.; Le Bras, M. Cardiac complications of halofantrine: a prospective study of 20 patients. *Trans. R. Soc. Trop. Med. Hyg.* **1995**, *89*, 430–433.
13. Ramaswamy, M.; Zhang, X.; Burt, H. M.; Wasan, K. M. Human plasma distribution of free paclitaxel and paclitaxel associated with diblock copolymers. *J. Pharm. Sci.* **1997**, *86*, 460–464.
14. Porter, C. J. H.; Charman, S. A.; Charman, W. N. Lymphatic transport of halofantrine in the triple cannulated anaesthetised rat model: effect of lipid vehicle dispersion. *J. Pharm. Sci.* **1996**, *85*, 351–356.

15. Redgraves, T. G.; Roberts, D. C. K.; West, C. E. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal. Biochem.* **1975**, *65*, 42−49.
16. Florence, A. T.; Halbert, G. W. Lipoproteins and microemulsions as carriers of therapeutic and chemical agents. In *Lipoproteins as carriers of pharmacological agents*; Shaw. J. M., Ed.; Marcel Dekker Inc.: New York, 1991; pp 141−174.
17. Humberstone, A. J.; Currie, G. J.; Porter, C. J. H.; Scanlon, M. J.; Charman, W. N. A simplified liquid chromatography assay for the quantitation of halofantrine and desbutylhalofantrine in plasma and identification of a degradation product of desbutylhalofantrine formed under alkaline conditions. *J. Pharm. Biomed. Anal.* **1995**, *13*, 265−272.
18. Shen, B. W.; Scanu, A. M.; Kezdy, F. J. Structure of human serum lipoproteins inferred from compositional analysis. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 837−841.
19. Meydani, M.; Cohn, J. S.; Macauley, J. B.; McNamara, J. R.; Blumberg, J. B.; Schaefer, E. J. Postprandial changes in the plasma concentration of α- and γ-tocopherol in human subjects fed a fat-rich meal supplemented with fat-soluble vitamins. *J. Nutr.* **1989**, *119*, 1252−1258.
20. Krishna, S.; ter Kuile, F. O.; Supanaranond, W.; Pukrittayakamee, S.; Teja-Isavadharm, P.; Kyle, D. E.; White, N. J. Pharmacokinetics, efficacy and toxicity of parenteral halofantrine in uncomplicated malaria. *Br. J. Clin. Pharmac.* **1993**, *36*, 585−591.
21. Sgoutas, D.; MacMahon, W.; Love, A.; Jerkunica, I. Interaction of cyclosporin A with human lipoproteins. *J. Pharm. Pharmacol.* **1986**, *38*, 583−588.
22. Glasson, S.; Zini, R.; D'Athis, P.; Tillement, J. P.; Boissier, J. R. The distribution of bound propranolol between the different human serum proteins. *Mol. Pharmacol.* **1980**, *17*, 187−191.
23. Chassany, O.; Urien, S.; Claudepierre, P.; Bastian, G.; Tillement, J. P. Binding of anthracycline derivatives to human serum lipoproteins. *Anticancer Res.* **1994**, *14*, 2353−2355.
24. Glasson, S.; Zini, R.; Tillement, J. P. Multiple human serum binding of two thienpyridinic derivatives, ticlopidine and PCR 2362, and their distribution between HSA, α-acid glycoprotein and lipoproteins. *Biochem. Pharmacol.* **1982**, *31*, 831−835.
25. Urien, S.; Albengres, E.; Comte, A.; Kiechel, J. R.; Tillement, J. P. Plasma protein binding and erythrocyte partitioning of nicardipine in vitro. *J. Cardiovasc. Pharmacol.* **1985**, *7*, 891−898.
26. Urien, S.; Claudepierre, P.; Meyer, J.; Brandt, R.; Tillement, J. P. Comparative binding of etretinate and acitretin to plasma proteins and erythrocytes. *Biochem. Pharmacol.* **1992**, *44*, 1891−1893.
27. Chamouard, J. M.; Barre, J.; Urien, S.; Houin, G.; Tillement, J. P. Diclofenac binding to albumin and lipoproteins in human serum. *Biochem. Pharmacol.* **1985**, *34*, 1695−1700.
28. Cenni, B.; Meyer, J.; Brandt, R.; Betschart, B. The antimalarial drug halofantrine is bound mainly to low- and high-density lipoproteins in human serum. *Br. J. Clin. Pharmacol.* **1995**, *39*, 519−526.

Acknowledgments

This work was supported, in part, by SmithKline Beecham Pharmaceuticals (U.K.). The authors thank Dr. John Horton for his interest and support of these studies, Dr. Andrew Humberstone for initial discussions, and M.P.M. gratefully acknowledges scholarship support provided by an Australian Postgraduate Award.

JS980152G